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Inhibition of Cuticular Lipid Biosynthesis in *Pisum sativum* by Thiocarbamates[1, 2]

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ABSTRACT

Treatment of slices of young pea leaves (*Pisum sativum*) with μM solutions of α-chlorallyl diethyldithiocarbamate, dichloroallyl diisopropylthiocarbamate, or S-ethyldipropylthiocarbamate resulted in inhibition of incorporation of [1-^{14}C]acetate into C_{31} alkane and C_{31} secondary alcohol, very little effect on the synthesis of C_{26} and C_{28} fatty alcohols, and an accumulation of ^{14}C in shorter chain cuticular lipids, particularly C_{22} acid. Higher concentrations of the thiocarbamates caused inhibition of synthesis of C_{26} and C_{28} fatty alcohols and an accumulation of label in C_{22} acid. Further increase in thiocarbamate concentration resulted in inhibition of C_{22} acid synthesis also. The three thiocarbamates at μM concentration also inhibited incorporation of [1-^{14}C]stearic acid specifically into C_{31} alkane and C_{31} secondary alcohol. These results suggest that thiocarbamates reduce cuticular lipid formation by a concentration-dependent inhibition of the various chain-elongating enzyme systems.

Certain thiocarbamates were reported to be inhibitory to wax production on foliage (4, 5, 12). Experimental evidence thus far available shows that fatty acid elongation is a key process involved in cuticular wax synthesis (2, 10). Recent observation that thiocarbamates inhibited synthesis of C_{20} to C_{24} fatty acids in germinating seeds (6) suggested a possible mechanism by which these herbicides might affect cuticular wax production. However, germinating seeds did not incorporate substantial amounts of labeled precursors into the typical surface lipid components. For example, alkanes, one of the major surface lipid components, were not labeled. Since elongation of fatty acid followed by decarboxylation seems to be the most likely mechanism for alkane synthesis, thiocarbamates should be expected to be inhibitors of alkane biosynthesis, if they are in fact inhibitors of the elongation process. In this paper, we describe the effects of several thiocarbamates on the synthesis of lipid components in the leaves of *Pisum sativum*. The experimental results presented strongly suggest that carbamates are potent inhibitors of chain elongation and, consequently, of alkane biosynthesis.

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MATERIALS AND METHODS

Materials. Pea plants (*Pisum sativum* var. Frosty) were grown as described before (1) and only the young expanding leaves near the plant apex were used. Sodium [1-^{14}C]acetate (55 c/mole) and [1-^{14}C]stearic acid (48 c/mole) were purchased from Amersham/Searle Corporation, Arlington Heights, Illinois. [^{14}C]Toluene for internal standard was purchased from New England Nuclear Corporation. [1-^{14}C]Stearic acid was dispersed in water with the aid of a few μg/ml of Tween-20 and sonication with a Biosonik III (needle probe at full power 2 × 20 sec.). α-Chloroallyl diethyldithiocarbamate and S-ethyldipropylthiocarbamate were obtained from Chemical Service Inc., Westchester, Pennsylvania, and dichloroallyl diissopropylthiocarbamate was obtained from Monsanto Chemical Company. The thiocarbamates were dispersed in water by sonication.

Incubation and Isolation of Products. Fresh young pea leaves were sliced (about 3 × 12 mm) and 250-mg portions were placed in 50-ml Erlenmeyer flasks containing sodium [1-^{14}C]acetate (182 nmoles, 10 μc) and appropriate amounts of the thiocarbamate in a total volume of 0.5 ml of water. After the contents of the flask were thoroughly mixed, the leaf slices were spread on the bottom of the flasks which were then incubated for 90 min at 30 C in a gyratory water bath shaker. At the end of the incubation period the tissue slices were transferred into a 150-ml sintered glass funnel and 100-ml of a 2:1 mixture of chloroform and methanol was added. After stirring the mixture for 20 sec, the solvent mixture was quickly removed by suction. The lipids were recovered from this solution according to the method of Folch *et al.* (3), and this fraction is referred to as surface lipids. The tissue slices were then extracted with a 2:1 mixture of chloroform and methanol for 2 hr, and the lipids recovered from this extract are referred to as internal lipids.

Fractionation of Lipids. Surface lipids were dissolved in 0.5 ml of chloroform and 10 μl were used for determination of radioactivity. Fifty μl were subjected to TLC on 0.5-mm layers of Silica Gel G with benzene as the developing solvent. The plates were sprayed with a 0.1% alcoholic solution of 2',7'-dichlorofluorescein and the components were marked under UV light. (In order to aid in the visualization of the components on the thin-layer plates, unlabeled surface lipids were added to the labeled material prior to chromatography.) Radioactivity in each fraction was determined by scintillation counting of the silica gel from appropriate areas as described before (7).

Wax ester fractions, isolated by TLC with benzene as the developing solvent, were refluxed with 14% BF_3 in methanol for 4 to 6 hr, and the products, isolated in the usual manner, were subjected to TLC with hexane-ethyl ether-formic acid

(40:10:1 v/v) as the developing solvent. The methyl ester and alcohol fractions were isolated and aliquots were assayed for ^{14}C. The lipids remaining in the origin of the thin layer chromatogram of the surface lipids (benzene as developing solvent) were also treated with BF_3-methanol, and the resulting methyl esters were isolated and subjected to radio gas-liquid chromatography. The alcohols derived from wax esters as well as free alcohols isolated from the surface lipid were acetylated overnight with a 2:1 mixture of acetic anhydride and pyridine at room temperature. The alcohol acetates recovered in the usual manner were subjected to radio gas-liquid chromatography.

Determination of Radioactivity. Radioactivity in liquid samples was determined by liquid scintillation counting with a Packard liquid scintillation spectrometer and a scintillation mixture containing 6 g of 2,5-diphenyloxazole and 125 mg *p*-bis(2-(5-phenyloxazolyl))benzene/1 of a 7:3 mixture of toluene and ethanol. Internal standards were always used to determine counting efficiency, which was usually about 70%. Counting was done with standard deviation less than 3%.

Table I. *Effect of Thiocarbamates on the Incorporation of [1-^{14}C]Acetate into Surface Lipids and Internal Lipids of Pea Leaves*

Concentration of Thiocarbamate	Incorporation of Radioactivity into Lipids					
	Avadex		EPTC		CDEC	
	Surface	Internal	Surface	Internal	Surface	Internal
μM	*dpm* × 10^{-6}					
0	2.44	4.27	2.84	5.61	3.54	6.16
1	2.00	4.39	3.34	5.36	3.10	5.56
3	1.71	3.61	2.29	5.20	3.13	5.13
6	1.64	4.80	1.77	4.91	2.14	3.93
9	1.53	4.84	1.79	4.69	1.69	4.91

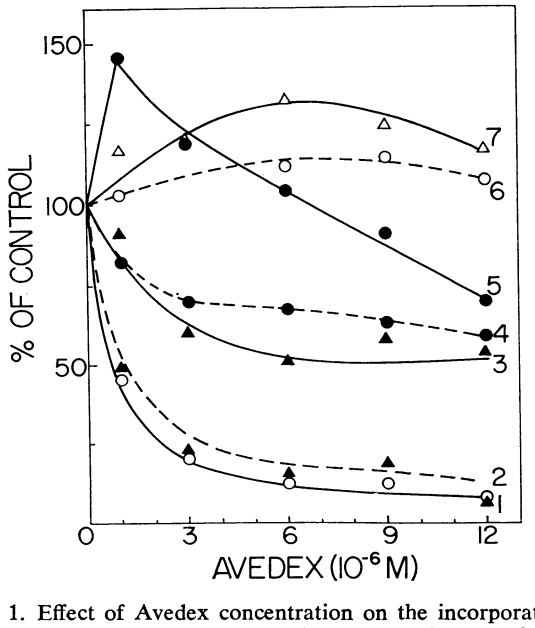

FIG. 1. Effect of Avedex concentration on the incorporation of [1-^{14}C]acetate into surface lipid fractions of pea leaves. The measurements were made as described under "Materials and Methods" 1: alkanes; 2: secondary alcohols; 3: primary alcohols; 4: total surface lipids; 5: wax ester; 6: internal lipids; 7: surface lipid origin (benzene solvent) fraction.

Radioactivity in gas-liquid chromatographic components was determined with a Barber Coleman radioactivity monitor attached to a Perkin-Elmer 800 gas chromatograph as described before (8).

Chromatography. Silica Gel G layers (0.5 mm or 1.0 mm), activated overnight at 110 C, were used for TLC with appropriate solvents indicated elsewhere. Gas-liquid chromatography was done with a coiled stainless steel column (180 × 0.625 cm o.d.) packed with 5% OV-1 on 80–100 mesh Gas Chrom Q. Alcohol acetates were analyzed with a column temperature of 260 C, while methyl esters were analyzed with temperature programming indicated under the figure legend.

RESULTS AND DISCUSSION

In order to seek a biochemical explanation for the observation that application of sublethal doses of thiocarbamate herbicides brought about reduction in foliar wax in plants (4, 5), we studied the effect of several thiocarbamates on conversion of [1-^{14}C]acetate into lipids of pea leaves. Usually incorporation was done for 90 min, during which time incorporation of label into lipids was linear. In this time period, about 30% of the exogenous acetate was incorporated into lipids, of which about one-third was in the surface lipid fraction. Since the incorporation varied somewhat from batch to batch, comparisons were made only among the samples of a given experiment. Results in Table I show that Avadex,[3] EPTC, and CDEC inhibited conversion of exogenous [1-^{14}C]acetate into surface lipids while incorporation of labeled acetate into internal lipids was less sensitive to the thiocarbamates. Since surface lipids are known to contain much longer aliphatic chains than the internal lipids (9, 10), these results agree with the suggestion that thiocarbamates inhibit chain elongation.

If the major effect of thiocarbamates is to inhibit chain elongation it might be expected that the synthesis of the very long chain components of the surface lipids would be more susceptible to the thiocarbamates than that of the shorter components. In order to examine this possibility, the surface lipids were further analyzed by TLC (Fig. 1). The synthesis of the longest aliphatic chains, the alkanes (mainly C_{31}) and the secondary alcohols (mainly C_{31}), was much more sensitive to Avadex than that of the other components. For example, at a concentration of Avadex which inhibited the synthesis of alkanes and secondary alcohols by 50%, incorporation into total surface lipids was inhibited only slightly (15%). The synthesis of the primary alcohol fraction, which is known to contain C_{26} and C_{28} (9, 11), was less sensitive to thiocarbamates than that of alkanes and secondary alcohols. With 3 μM Avadex, incorporation of [1-^{14}C]acetate into alkanes and secondary alcohols was inhibited about 80%, while incorporation into primary alcohol was inhibited only about 35%. The effect of Avadex on wax ester synthesis was quite different in that at low concentrations of Avadex there was a significant stimulation of incorporation of label into wax esters. As the concentration of Avadex increased, the extent of stimulation decreased, and finally higher concentrations of Avadex inhibited wax synthesis also. Incorporation of acetate into lipids, which remained in the origin of the thin layer chromatograms of surface lipids, was also somewhat stimulated by fairly low concentrations of Avadex.

In general, the effects of the other two thiocarbamates used in the present investigation, EPTC and CDEC, on the in-

[3] Abbreviations: Avedex: dichloroallyl diisopropylthiocarbamate; EPTC: S-ethyldipropylthiocarbamate; CDEC: α-chloroallyl diethyldithiocarbamate.

Table II. *Effect of Thiocarbamates on the Incorporation of [1-^{14}C]Acetate into Components of Surface Lipids in Pea Leaves*

In the control experiment containing no thiocarbamate about 30%, 10%, 3%, 7%, and 35% of the ^{14}C of the total surface lipids were found in hydrocarbon, secondary alcohol, wax ester, primary alcohol, and origin fractions, respectively.

Incorporation	Concentration of Carbamate			
	1 μM	3 μM	6 μM	9 μM
	% of control			
Hydrocarbon				
EPTC	79	44	16	11
CDEC	60	34	9	4
Secondary alcohol				
EPTC	81	41	17	14
CDEC	76	55	17	6
Primary alcohol				
EPTC	114	116	92	78
CDEC	90	127	68	36
Wax Ester				
EPTC	123	183	159	149
CDEC	127	209	104	74
Origin				
EPTC	113	157	116	133
CDEC	104	141	88	68

corporation of [1-^{14}C]acetate into thin layer chromatographic fractions of the pea leaf surface lipids were similar to those observed with Avadex (Table II). It seems that CDEC was slightly more effective than EPTC in inhibiting the synthesis of wax esters and the lipids in the origin. For example, 9 μM CDEC inhibited incorporation of acetate into wax esters and lipids in the origin by about 35%, while EPTC did not show any inhibition at this concentration. Except for this relatively minor difference, the three thiocarbamates showed similar patterns of inhibition of the surface lipids in pea leaves. These results show that the thiocarbamate herbicides can cause specific inhibition of certain components of the surface lipids. Such inhibition would result in altered appearance of surface lipids and probably increased penetration of chemicals applied after carbamate treatment, as has been observed before (4, 5).

In order to investigate the possibility that the major effect of thiocarbamates on surface lipid synthesis is inhibition of chain elongation, all classes of surface lipids were further examined. Since ^{14}C accumulated in wax esters while incorporation of [1-^{14}C]acetate into free primary alcohols was inhibited, it appeared likely that the acyl portion of the wax ester should contain the accumulated label. In order to test this possibility, the amounts of ^{14}C in the acyl portion and alcohol portion were determined. As the concentration of thiocarbamates increased, the proportion of the ^{14}C contained in the acyl portion of the wax increased (Table III). Taking the accumulation of label in the wax ester fraction into consideration, significant inhibition of incorporation of acetate into the alcohol portion of the wax was observed only at or above 6 μM CDEC and 9 μM EPTC. Similar effects on free fatty alcohol fractions were also observed (Table II). Radio gas liquid chromatographic analysis of the free alcohols and alcohols derived from wax showed that under all conditions C_{26} was the major labeled alcohol followed by C_{28}. It appeared that thiocarbamates tended to inhibit the labeling of C_{28} a little more than that of C_{26}.

Since the inhibition of synthesis of very long chain alkanes and secondary alcohols was accompanied by accumulation of label mainly in the acyl portion of the wax (with 3–6 μM inhibitor), the chain lengths of the accumulated acids were examined by radio gas-liquid chromatography (Table IV). The major labeled fatty acids in the wax esters of untreated tissue were C_{20}, C_{22}, and C_{24}, with smaller amounts of C_{16}, C_{18}, C_{21}, and C_{23}. The major change brought about by the three thiocarbamates was a dramatic increase in the C_{22}. For example, as the concentration of EPTC increased from 1 to 12 μM, the C_{22} acid became the major acid containing about 60% of the total ^{14}C of the acyl portion of the wax (Table IV). Similar changes were observed with Avadex and CDEC (data not shown).

The radioactive free fatty acids of the surface lipids remained in the origin of the thin layer chromatograms (benzene as developing solvent). Since these acids are likely to be related to the acyl portion of the wax, the effect of thiocarbamates on chain length patterns might be reflected in these fatty acids. In order to test this possibility, the methyl esters generated by treatment of the lipids in the origin with BF_3-methanol were subjected to radio gas-liquid chromatography. Since this fraction would have contained some internal polar lipids, the presence of C_{16} and C_{18} should be expected, and they were two of the major labeled components. However, the thiocarbamates did not significantly affect the synthesis of these acids. The longer components were C_{20}, C_{22}, C_{24}, and C_{26}, with smaller

Table III. *Effect of Thiocarbamates on the Distribution of Label from [1-^{14}C]Acetate between the Acid and Alcohol Portions of the Wax Ester of Pea Leaves*

A portion of the wax ester fraction was subjected to methanolysis with BF_3-methanol and products were analyzed by TLC.

Concentration of Thiocarbamate	Radioactivity (Acid/Alcohol)	
	EPTC	CDEC
μM	ratio	
0	1.33	0.84
1	1.04	0.90
3	1.01	1.36
6	1.43	1.84
9	1.65	2.22
12	1.63	2.33

Table IV. *Effect of EPTC Concentration on the Chain Length Distribution of the Labeled Fatty Acids Derived from the Wax Esters of Pea Leaves*

The methyl esters obtained by BF_3-methanol treatment of wax ester fractions were subjected to radio gas-liquid chromatography on a 5% OV-1 column held at 200 C for 6 min and then temperature programmed up to 293 C at 7.5 C/min.

Concentration of EPTC	Radioactivity Distribution						
	C_{16}	C_{18}	C_{20}	C_{21}	C_{22}	C_{23}	C_{24}
μM	%						
0	8.5	8.3	19.9	6.8	31.2	4.3	21.3
1	3.1	7.6	19.1	8.0	44.7	1.8	15.8
3	5.4	8.0	12.8	7.5	56.1		10.3
6	5.6	4.4	11.7	5.4	48.0	6.0	19.2
9	8.1	4.5	11.9	7.0	48.4	5.7	13.8
12	8.1	6.3	16.4	4.0	59.6	4.3	9.4

Table V. *Effect of EPTC on Incorporation of [1-^{14}C]Acetate into the Very Long Chain Acids of the Surface Lipids*

The methyl esters of fatty acids derived from the surface lipids which remained in the origin (benzene solvent) were analyzed by radio gas-liquid chromatography. Since the C_{16} and C_{18} acids contained in this fraction most probably originated from the internal lipid contamination, only the very long chain acids are included in this calculation.

Concentration of EPTC	Radioactivity Distribution			
	C_{20}	C_{22}	C_{24}	C_{26}
μM	%			
0	12.6	28.9	18.5	40.2
3	15.2	33.1	20.1	31.6
6	11.9	37.5	24.6	26.0
12	13.0	49.1	19.8	18.0

Table VI. *Effect of Thiocarbamates on the Incorporation of [1-^{14}C]Stearic Acid into Surface Lipid Components in Pea Leaves*

[1-^{14}C]Stearic acid (2 μc, 42.5 nmoles) was incubated with 200 mg of pea leaves in a total volume of 0.5 ml of water for 30 min at 30 C. In the control experiment without any thiocarbamate 211,000, 33,000, 26,000, and 41,000 dpm were obtained in the hydrocarbon, secondary alcohol, wax ester, and primary alcohol fractions, respectively. Values in this table were calculated considering each of these values as 100%.

Concentration	Incorporation			
	Hydrocarbon	Secondary alcohol	Wax ester	Primary alcohol
μM	% of control			
Avadex				
3	72.2	91.4	93.5	156.0
12	24.8	40.5	94.8	70.4
EPTC				
3	56.9	74.0	96.2	100.3
12	20.7	31.6	98.6	71.3
CDEC				
3	42.7	41.5	119.7	131.0
12	18.2	29.8	101.3	125.0

amounts of C_{28}, and the thiocarbamates significantly affected the distribution of label among these acids (Table V). As the concentration of EPTC increased, the proportions of label in C_{26} decreased and that in C_{22} increased. Further increase in EPTC also inhibited formation of C_{22}. Similar changes were observed with CDEC and Avadex. Gas-liquid chromatographic analysis of the methyl esters prepared from internal lipids showed that C_{16} and C_{18} were the major labeled acids with very little longer chain acids (data not shown). Carbamate treatment under the conditions used here did not change the chain length distribution pattern of internal lipids significantly.

The results discussed thus far show that the three thiocarbamates examined inhibited the longest (C_{31}) components of the surface lipids at the lowest concentrations. In fact they constitute the most potent inhibitors of alkane biosynthesis known thus far. As the concentration increased, the synthesis of C_{26} and C_{28} components (C_{26} and C_{28} alcohols and C_{26} acids) was inhibited. At the same time labeled C_{22} acid accumulated. The reason for this specific increase is unknown. It is possible that the various classes of elongated chains are produced by different elongating enzyme systems. If so, inhibition of one system, such as that which produces C_{31}, by a given concentration of a thiocarbamate could provide increased amounts of elongating agent (malonyl-CoA) for other systems, such as that which synthesizes C_{22}. It is also possible that accumulation of C_{22} acid represents a partially elongated product.

The effect of the three thiocarbamates on the incorporation of [1-^{14}C]stearic acid into surface lipids was also studied. Although stearic acid is known to be more efficiently converted into alkanes and other surface lipids than other precursors (8), the effects of thiocarbamates on the incorporation of this acid into the surface lipids were similar to those observed with [1-^{14}C]acetate (Table VI). All three thiocarbamates inhibited incorporation of [1-^{14}C]stearic acid into alkanes and secondary alcohols most severely with a little or no inhibition of incorporation into other components. Since chain elongation is the major type of reaction involved in the conversion of stearic acid into the surface lipids, these results are in agreement with the hypothesis that thiocarbamates inhibit surface lipid synthesis by inhibiting the chain elongation process.

The observation that thiocarbamates, which are known to inhibit chain elongation in germinating seeds (6), also inhibit alkane synthesis is consistent with the hypothesis that alkanes are synthesized by elongation followed by decarboxylation. Inhibition of synthesis of alkanes by thiocarbamates was accompanied by similar inhibition of secondary alcohol formation. This observation is in agreement with the previously published data which suggested that secondary alcohols are derived from alkanes (10). The recent observation that thiocarbamates inhibit mycolic acid synthesis in mycobacteria (K. Takayama, private communication) suggests that the elongation systems in higher plants and bacteria may be similar.

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